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Partial Reverse Transcripts in Virions from Human Immunodeficiency and Murine Leukemia Viruses

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Reverse transcription of the retroviral genome is thought to start after virions enter target cells. Purified preparations of human immunodeficiency virus were found to contain virus-specific DNA, detectable by polymerase chain reaction amplification. This DNA resulted from reverse transcription in newly assembled virus particles and not from contamination by cellular DNA, because virions contained a striking excess of early versus late transcripts and because the accumulation of these products was sensitive to 3′-azido-3′-deoxythymidine (zidovudine) treatment of producer cells. A similar observation was made with murine amphotropic retrovirus particles. It is therefore likely that all retroviruses contain partial reverse transcripts.

Retroviruses are characterized by the ability to convert a diploid, single-stranded RNA genome into a double-stranded DNA copy. Traditionally, their replicative cycle is thought to proceed in two separate phases (35). The first phase includes the entry of the virion into the cell cytoplasm, the synthesis of proviral DNA, and its integration into the host cell chromosome. The second phase results from the expression of viral genes and includes the synthesis of viral RNA and proteins, their assembly at the cell membrane, and the release of new viral particles. The synthesis of proviral DNA is mediated by virus-encoded reverse transcriptase (RT) (2, 32), which contains RNA- and DNA-directed DNA polymerase and ribonuclease H activities (35). Additional factors are apparently necessary, because only the early phases of reverse transcription can be obtained in "reconstructed" systems, using purified RT (35). Most of these factors, however, seem to be present in viral particles, since detergent-disrupted virions can be induced to complete the synthesis of extended and even biologically active reverse transcripts, as long as the proper amounts of nucleotides and specific divalent cations are provided (5, 10, 24–26).

Shortly after it had been shown that the principal nucleic acid of retroviruses was a single-stranded RNA, small amounts of DNA were found in purified preparations of avian and murine retroviruses (4, 14, 22, 23). Subsequent analyses performed on Rous sarcoma virus, however, did not demonstrate any complementarity between this DNA and viral RNA. In addition, substitution of bromodeoxyuridine for thymidine in virion-associated DNA, followed by treatment with visible light, failed to affect the infectivity of the virus (15). On that basis, it is currently held that DNA contained in retroviral particles is a cellular contaminant, devoid of any function in the life cycle of the virus. Also, it is believed that reverse transcription is not initiated before virions uncoat in the target cells because of a lack of essential, cell-derived factors in the virion (34) and/or the need for structural changes in the viral core that take place at the time of uncoating.

Besides retroviruses, two groups of DNA viruses replicate their genome through reverse transcription: the hepadnaviruses, which include hepatitis B virus, and the caulimoviruses, which are plant DNA viruses. However, in striking contrast to retroviruses, hepadnaviruses and caulimoviruses release particles that contain an almost completed reverse transcription product (for a review, see reference 18). In these latter viruses, the conditions required for DNA synthesis are obviously met before virions are released.

The polymerase chain reaction (PCR) has revolutionized the analyses of a number of biological phenomena, by increasing the ability to detect specific nucleic acid sequences considerably. Because of its exquisite sensitivity, this technique has become the tool of choice in the study of viruses, including the human immunodeficiency virus (HIV). PCR is used in clinical studies to detect and monitor HIV infection in patients. It is also extensively utilized in vitro, for instance, to analyze specific steps of the retroviral life cycle. PCR appears to be particularly valuable for dissecting the early events of infection, before viral gene expression is measurable by more conventional techniques. These early steps can be examined by amplifying DNA extracted from freshly infected cells, using virus-specific primers (11, 17, 30, 31, 37). Because reverse transcription is not believed to start before viral entry, the products obtained are thought to reflect the internalization of the virions and the synthesis of proviral DNA in the cytoplasm of newly infected cells. However, such a concept needs to be reconsidered, as the results presented here demonstrate the presence of virus-specific DNA in particles from two distinct retroviruses, HIV and murine leukemia virus (MLV).

MATERIALS AND METHODS

Cells and viruses. All cells were grown in RPMI medium supplemented with 10% fetal calf serum. H9(R7) are H9 cells chronically infected with a derivative of the HIV type 1 (HIV-1) HXB2D isolate (27), with a full-length *nef* coding region as described previously (13). H9(ΔE-neo) are H9 cells constitutively expressing an *env*-defective version of R7, as previously described (8, 33). Briefly, the ΔE-neo construct was obtained by introducing an 8-mer *Mlu*I linker at position 6833 of the R7 genome in plasmid R7-HXB2 (25), resulting in a translational frameshift in the 5′ portion of *env*; in addition, the sequence coding for neomycin phosphotransferase was inserted between the initiation codon for Nef and the proximal border of the 3′ long terminal repeat. H9(ΔE-neo) cells were generated by electroporation with ΔE-neo and selection with 1 mg of G418 per ml. H9(ΔE-neo) produces large amounts of naked HIV-1 particles, as verified by Western

blotting (immunoblotting) and electron microscopy (not shown). As predicted, when these cells are cocultivated with other cells expressing high levels of CD4, like CEM or C8166 cells, no fusion is observed. ACH2 is a HIV-1-infected human T-cell line which contains a single copy of the provirus (9) and minimal quantities of unintegrated DNA (3). CEM-1B (a kind gift from D. Spector, University of California at San Diego) are CEM cells infected with a murine amphotropic retrovirus (29). Cellular DNA was prepared by incubation of washed cells in a solution consisting of 100 mM NaCl, 25 mM EDTA, 10 mM Tris (pH 8), 0.5% sodium dodecyl sulfate (SDS), and 0.1 mg of proteinase K per ml at 50°C overnight, followed by phenol-chloroform extraction and ethanol precipitation, as previously described (1).

Virion preparation. Virions were prepared from H9(R7), H9(ΔE-neo), and CEM-1B cells by filtration of 30 to 60 ml of cell supernatant through a 0.45-μm-pore-size nitrocellulose membrane, followed by ultracentrifugation at 23,000 rpm in an SW28 rotor (Beckman) for 2.5 h at 4°C; the pellet was resuspended in phosphate-buffered saline (PBS). When indicated, samples of resuspended pellet (100 μl) were fractionated by gel-exclusion chromatography on 2-ml columns of Sephacryl S-1000 (Pharmacia), as previously described (19, 20). Elution was done with 2 ml of PBS, and 3-drop fractions (average, 160 μl) were collected. Concentrations of p24 protein were determined in an enzyme-linked immunosorbent assay ELISA (Dupont). Virus disruption before PCR analysis was achieved by adding Nonidet P-40 (NP-40) to a final concentration of 0.5%, before or after incubation for 5 min in a boiling waterbath.

Nuclease digestions. Virus preparations were digested with 2 μg of DNase I per ml in the presence of 10 mM $MgCl_2$, before or after disruption in 0.5% NP-40. DNase was inactivated before PCR analysis by boiling the samples for 15 min.

PCR amplification. All primers were synthesized using a PCR-Mate 391 DNA synthesizer (Applied Biosystems), following the manufacturer's instructions. The sequences of the HIV-specific primers are as follows (positions of complementary nucleotides in the HIV-1 HXB2D sequence are indicated in parentheses): U3, CACACACAAGGCTACT TCCCT (57 to 77); R, GGCTAACTAGGGAACCCACT GCTT (496 to 516); U5, CTGCTAGAGATTTTCCACACT GAC (635 to 612); 5NC, CCGAGTCCTGCGTCGAGAG AGC (698 to 677); Gag1, GCGAGAGCGTCAGTATTAA GCGGGG (795 to 819); Gag2, GGCTGACCTGATTGCTGT GTCCTG (1176 to 1153); Vif1, GGGAAAGCTAGGGGATG GTTTTAT (5136 to 5159); Vif2, CAGATGAATTAGTTGGT CTG (5366 to 5347); Nef1, CCTGGAATTCGTGTGATTGG ATGGCCTACTG (8821 to 8841; contains some noncomplementary sequence at its 5′ end); and Nef2, GTGGCTAAGA TCTACAGCTG (9062 to 9043). Primers specific for Moloney MLV sequences were also generated, complementary to the long terminal repeat and the 5′ noncoding region of Moloney MLV (28): MU3, CCTGTAGGTTTGGCAAG CTAGCT (nucleotides 7830 to 7852 of the MLV genome, linear map); MR, ATACACGGGTACCCGGGCGACTC (44 to 22); and M5NC, GCTGGCCAGCTTACCTCCCGGT (219 to 198). PCR amplifications were performed using 50 pmol of each primer, in a 50-μl reaction mixture containing 200 μM each of the four deoxynucleoside triphosphates, 50 mM KCl, 10 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 10 ng of genomic DNA from uninfected H9 cells (unless indicated otherwise), and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus). The reaction mixture was overlaid with 50 μl of mineral oil and subjected to 30 cycles in a Perkin-Elmer thermocycler (denaturation for 1 min at 94°C, primer annealing for 2 min at 55°C, and polymerization for 3 min at 72°C), with a final 7-min-long extension step at 72°C.

Southern blot hybridization. Amplified products were analyzed by electrophoresis through a 1.2% agarose gel and transferred to nylon membrane (Zetabind; Cuno), in 0.4 N NaOH–2 mM EDTA. DNA was cross-linked to the membrane with UV light (12 μJ), using a Stratalinker (Stratagene). Filters were prehybridized for 2 h at 42°C in a solution consisting of 50% formamide, 4× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 0.1% SDS, 1× Denhardt's solution, and 100 μg of herring sperm DNA (Sigma) per ml. Hybridizations were done overnight in the same buffer at 42°C, using ^{32}P-labeled probe (0.5×10^6 cpm/ml). Probes were generated by random oligonucleotide labeling with a random primer kit (Prime It; Stratagene), following the manufacturer's instructions. The template used to generate the HIV-1-specific probe was a (*Hpa*I-*Xba*I) fragment from plasmid R7-HXB2 (27), containing the entire proviral sequence. The MLV-specific probe was similarly obtained by using a (*Hin*dIII-*Bam*HI) fragment from plasmid pGD′ (7), corresponding to the proximal 930 nucleotides of the MLV sequence. After hybridization, filters were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS twice briefly at room temperature and once at 65°C for 20 min, washed in 1× SSC and then in 0.5× SSC at 65°C for 20 min, and exposed to X-ray film, with or without an intensifying screen.

RESULTS

PCR amplification of DNA from freshly infected cells is an attractive method for studying retroviral entry, because it is expected that reverse transcription follows virus internalization. To analyze the early events of HIV-1 infection through this approach, a number of HIV-1-specific primers were selected on the basis of the accepted model for reverse transcription of retroviral DNA (35) (Fig. 1).

Virus-specific DNA in the supernatant of HIV-1-infected cells. Before attempting to study HIV-1 entry by PCR, a preliminary control was performed to ensure that no virus-specific DNA was associated with HIV-1 virions. Viral supernatant from the chronically infected H9(R7) cell line was filtered and purified by ultracentrifugation, and the pellet was lysed in 0.5% NP-40, before or after incubation for 10 min in a boiling waterbath. An aliquot of the lysate, corresponding to 1 ng of p24 antigen, was then submitted to PCR amplification, using HIV-1-specific primers, and the reaction products were analyzed by agarose gel electrophoresis (Fig. 2). Surprisingly, a strongly positive signal was obtained, readily visible by ethidium bromide staining of the gel (lanes 2 and 3). A similar result was obtained from supernatants of several other HIV-infected cell cultures (not shown). This finding triggered a succession of experiments, described below.

The viral DNA is contained within particles. To differentiate DNA associated with viral particles from DNA potentially released by lysed cells, virions were purified by gel-exclusion chromatography on 2-ml Sephacryl S-1000 columns. As previously described (19, 20), more than 90% of p24 activity is eluted from such columns with the peak of infectivity (not shown). Fractions (160 μl) were harvested and analyzed by ELISA for the presence of p24 viral antigen. The peak of p24 activity was recovered from an elution volume between 480 and 960 μl (Fig. 3, top, fractions

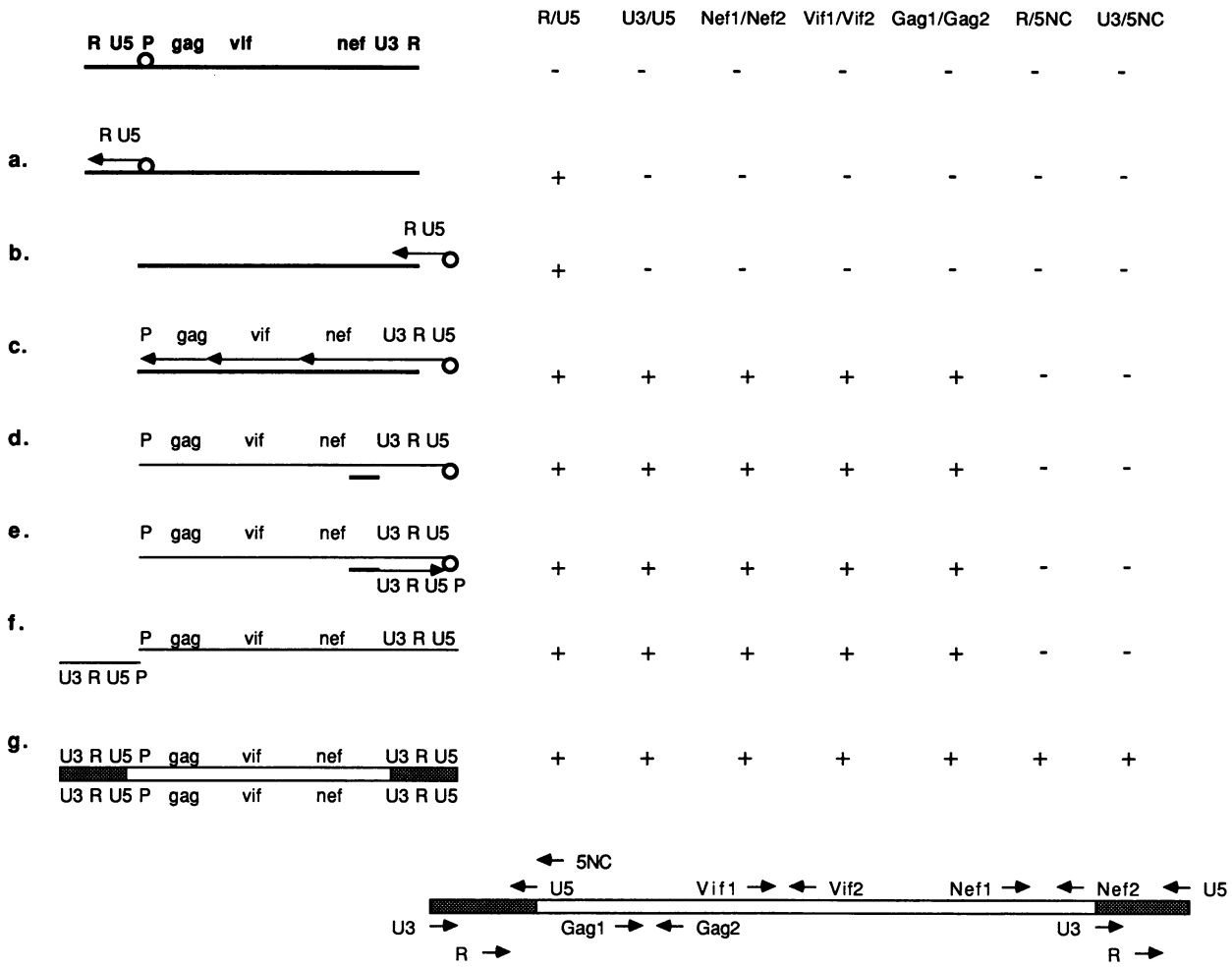

FIG. 1. Selection of PCR primers, based on the accepted model of reverse transcription. The various steps involved in the reverse transcription of retroviral RNA are schematically represented on the left side. Thick line, viral RNA; thin line, DNA; bold circle, tRNA primer; P, primer-binding site. Arrowheads indicate the direction of synthesis. The accepted model for reverse transcription includes a succession of the following steps: initiation from a tRNA primer, synthesis of DNA complementary to the 5′ end of the viral RNA (minus-strand strong-stop DNA) (a), first template switching (b), elongation of the minus strand up to the primer-binding site (c and d), synthesis of the plus strand 3′ end from a polypurine tract located at the 5′ end of U3 (plus-strand strong-stop DNA) (e), second template switching (f), and completion of the double-stranded DNA molecule (g). The locations of the various PCR primers and their predicted abilities to amplify the various intermediates of reverse transcription are shown on the right side. Primer pair R/U5 was designed to detect the earliest RT products (minus-strand strong-stop DNA). With primer pairs U3/U5, Nef1/Nef2, Vif1/Vif2, and Gag1/Gag2, a specific PCR product was expected only if a minus-strand DNA of increasing length had been synthesized after the first template switching. Finally, primer pairs R/5NC and U3/5NC, which flank the primer-binding site, were predicted to amplify only late RT products, synthesized after the second template switching.

3 to 5). Aliquots from each fractions were submitted to PCR analysis, using HIV-specific primers. The result showed that the early fractions with peak levels of p24 antigen were the ones containing significant levels of virus-specific DNA (Fig. 3, bottom). In a parallel control, DNA purified from infected cells was loaded on a similar column and the harvested fractions were analyzed by PCR. A positive signal was recovered as a broad peak, at an elution volume between 960 to 2,080 μl (not shown). This confirmed that the HIV-specific DNA contained in the purified supernatant of infected cells was associated with viral particles. To determine whether this DNA was contained within nuclease-resistant structures or rather simply adsorbed at the virion surface, virus preparations were exposed to DNase I, before or after treatment with NP-40 (Fig. 4). The result showed that DNase digestion of intact virions did not significantly affect the amount of DNA detected (compare lanes 2 and 3), whereas prior disruption of the particles with NP-40 abolished the PCR signal (lane 4). RNase treatment had no effect (not shown). It was therefore concluded that virus-specific DNA is contained within HIV particles.

The HIV-1 particle-associated DNA results from partial reverse transcription of the viral RNA in newly assembled particles. The virus-specific DNA detected in virions could have resulted from the random packaging of DNA from infected cells, rather than from de novo synthesis in newly assembled virus particles. Two experiments were performed to test this hypothesis. First, HIV-1 virions purified by chromatography were analyzed by semiquantitative PCR, using primer pairs specific for the various stages of reverse

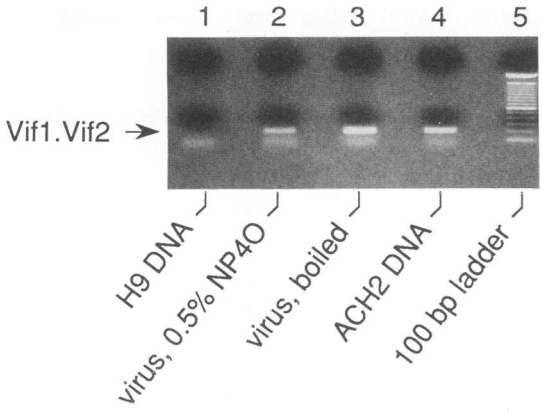

FIG. 2. PCR analysis of pelleted HIV-1 virions. Virions purified by ultracentrifugation from the supernatant of chronically infected H9(R7) cells were analyzed by PCR amplification, using primer pair Vif1/Vif2. The product was electrophoresed through a 1.2% agarose gel containing ethidium bromide and visualized with UV light. Lanes: 1, uninfected H9 DNA (10 ng); 2, virions (1 ng of p24) lysed in 0.5% NP-40; 3, virions (1 ng of p24) boiled for 10 min; 4, ACH2 DNA (10 ng); 5, molecular size markers (100-bp ladder).

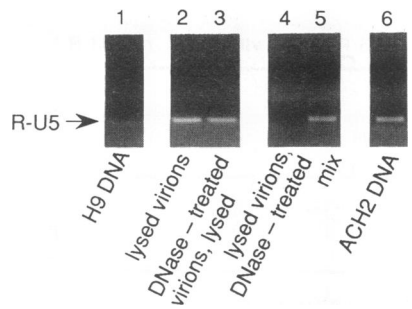

FIG. 4. Nuclease sensitivity of HIV-1 virion-associated DNA. Particles purified from the supernatant of H9(R7) cells were digested with 2 μg of DNase I per ml, before or after disruption with 0.5% NP-40, and submitted to PCR analysis, after inactivating the DNase by boiling the samples for 15 min. PCR products were electrophoresed through an agarose gel and visualized by ethidium bromide staining. The specificity of the signal was verified by Southern blot hybridization (not shown). Lanes: 1, uninfected H9 DNA (10 ng); 2, virions lysed in 0.5% NP-40; 3, virions treated with DNase I and then lysed in 0.5% NP-40; 4, virions lysed in 0.5% NP-40 and then treated with DNase I; 5, lysed virions treated with DNase I, mixed with undigested virions, and then submitted to PCR analysis, to ascertain that the DNase had been inactivated; 6, ACH2 DNA (10 ng).

transcription, to compare the abundance of early versus late RT products (Fig. 5). For each primer pair, limiting dilutions of ACH2 DNA were utilized as controls; in each case, the signal obtained from virions was compared with that yielded by amplification of the cellular DNA, to take into account the relative efficiencies of the various primer pairs. Several dilutions were performed in all cases, so that products could be compared in the linear range of the assay. The result demonstrated that primer pair R/U5, predicted to amplify all molecules which included the earliest DNA synthesized, before the first template switching, yielded approximately 10 times more product than primer pair Nef1/Nef2, which could amplify only DNA synthesized after the switch: with primers R/U5, an aliquot of virions corresponding to 100 pg of p24 (Fig. 5, lane 2) gave a signal at least equal to that recovered from 10 ng of ACH2 DNA (lane 6); in contrast, with the Nef1/Nef2 primer pair, a lower yield was obtained from 10 times more virions (compare lanes 1 and 6). Primer pair Vif1/Vif2, specific for later products, also gave a weaker signal. That virions contain more early than late reverse transcripts was most convincingly demonstrated by using primer pairs U3/5NC and R/5NC, which flank the primer-binding site, and are expected to detect only DNA synthesized after the second template switching: these clearly yielded the lowest amount of product (Fig. 6).

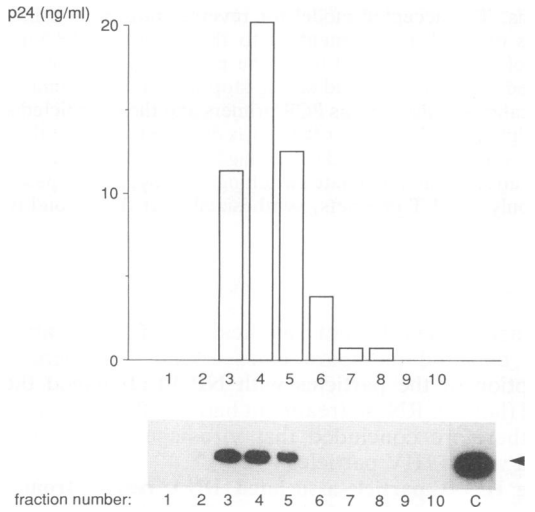

FIG. 3. PCR analysis of chromatography-purified HIV-1 virions. Viral pellet from the supernatant of H9(R7) cells was fractionated by gel-exclusion chromatography on a 2-ml Sephacryl S-1000 column collecting 160-μl fractions. The p24 content of each fraction was determined by ELISA. An aliquot was submitted to PCR amplification, using the Vif1/Vif2 primer pair; the product was analyzed by Southern blot hybridization. Fraction number 1 has an elution volume between 160 and 320 μl. Fraction C consists of H9(R7) DNA.

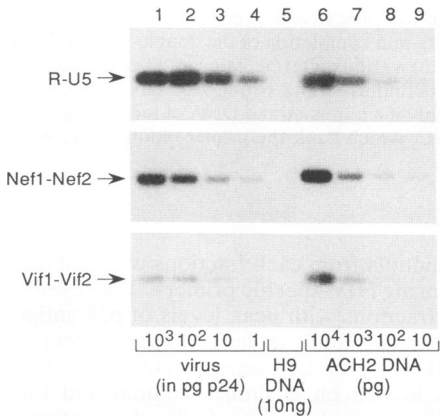

FIG. 5. Comparison of the various reverse transcription intermediates present in virions. Serial 10-fold dilutions of HIV virions purified by gel-exclusion chromatography were submitted to PCR amplification, using primers specific for some of the intermediates of reverse transcription, as described in the legend to Fig. 1. Dilutions of ACH2 DNA, which contains one copy of proviral DNA per genome, were used as controls; in all cases, 10 ng of DNA from uninfected H9 cells was used to equalize the amounts of DNA present in the reaction mixtures.

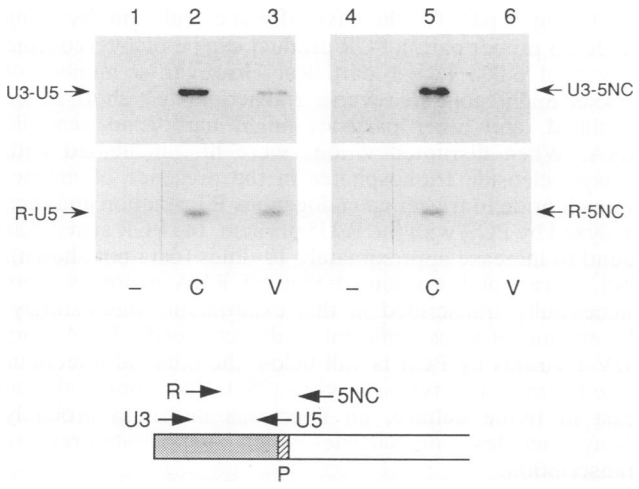

FIG. 6. Rarity of late reverse transcription products in HIV-1 virions. Lysate from column-purified HIV-1 particles, as shown in Fig. 3, was submitted to PCR amplification, using primer pairs specific for different stages of reverse transcription. R/U5 and U3/U5 (lanes 1 to 3) can detect all molecules which include sequences synthesized before or immediately after, respectively, the first template switching; R/5NC and U3/5NC (lanes 4 to 6) can amplify only late RT products, synthesized after the second jump. Lanes: 1 and 4, 10 ng of uninfected H9 DNA (–); 2 and 5, 10 ng of ACH2 DNA (C); 3 and 6, virions (V), 1 ng of p24. P, primer-binding site.

To further demonstrate that virus-associated DNA represented reverse transcription intermediates, chronically infected H9(R7) cells were treated for 3 days in medium containing 5 μM 3′-azido-3′-deoxythymidine (zidovudine; AZT), added fresh daily, before purifying virions released over 24 h. AZT was predicted to block the elongation of newly synthesized reverse transcripts. Virions from AZT-treated cells were then compared, by semiquantitative PCR, to viruses obtained from mock-treated infected cells (Fig. 7). The result showed that AZT treatment resulted in a striking reduction in the amount of elongated minus-strand DNA detected in particles. The experiment was repeated two more times, growing the H9(R7) cells in AZT-containing medium for up to 6 days, with a similar result.

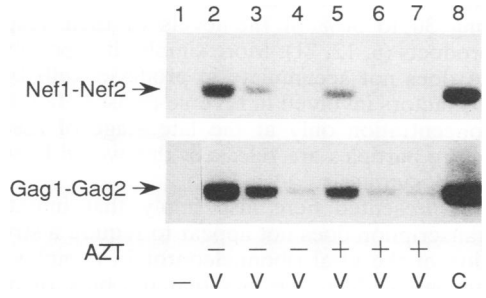

FIG. 7. AZT sensitivity of virus-associated DNA. Viral supernatants from H9(R7) cells mock treated (lanes 2 to 4) or grown for 3 days in medium containing 5 μM AZT (lanes 5 to 7) were purified by gel-exclusion chromatography. Aliquots from peak viral fractions were normalized for p24 content, and 10-fold dilutions were analyzed by PCR and Southern blot hybridization. Lanes: 1, uninfected H9 DNA. lanes 2 to 7, virus (V) normalized to 200 (lanes 2 and 5), 20 (lanes 3 and 6), and 2 (lanes 4 and 7) pg of p24; 8, H9(R7) DNA (C).

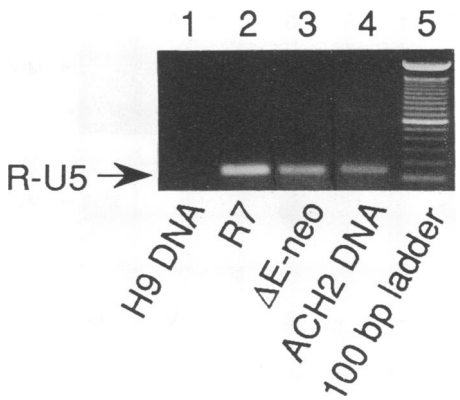

FIG. 8. PCR analysis of particles from cells transduced with an Env-defective HIV-1 provirus. Particles were purified from the supernatants of H9(R7) and H9(ΔE-neo) cells, and an aliquot corresponding to 200 pg of p24 antigen was submitted to PCR analysis, using the R/U5 primer pair. The product was analyzed by agarose gel electrophoresis and ethidium bromide staining. Its specificity was verified by Southern blot hybridization (not shown). Lanes: 1, uninfected H9 DNA (10 ng); 2, wild-type virions; 3, envelope-defective virions; 4, ACH2 DNA (10 ng); 5, molecular size markers (100-bp ladder).

The remote possibility that the virus-specific DNA was contained in viral cores, rather than in virions, still existed. Despite the down-regulation of its receptor by the virus envelope, a significant level of reinfection takes place in cells productively infected with retroviruses. Although no obvious cytopathic effect was observed in H9(R7) cells, one could not exclude the possibility that viral cores, internalized by reinfection and having initiated reverse transcription, were released by damaged cells, accounting for the DNA associated with particles. To rule out this possibility, virions from cells showing no reinfection were therefore analyzed (Fig. 8). The cell line H9(ΔE-neo) produces HIV-1 particles that are noninfectious, because they are devoid of envelope. Accordingly, no unintegrated viral DNA can be detected in these cells by PCR, reflecting the absence of newly internalized cores (not shown). PCR analysis of particles purified from the supernatant of H9(ΔE-neo) revealed the presence of virus-specific DNA (Fig. 8, lane 3), as in wild-type virions (lane 2). On that basis, it can be inferred that the partial reverse transcripts detected in the supernatant of HIV-infected cells are contained in virions and are not secondary to the release of cores by damaged cells.

Reverse transcription intermediates in MLV particles. Lentiviruses, including HIV, are characterized by an unprecedented genomic complexity and biological behavior, compared with classical retroviruses like MLV. It was therefore interesting to determine whether the presence of DNA in viral particles is unique to lentiviruses. To answer this question, viral particles were purified from the supernatant of CEM-1B, a human T-cell line chronically infected with a murine amphotropic retrovirus (29). The lysed virions were analyzed by PCR, using MLV-specific primers. This revealed that MLV particles, like HIV, contained significant amounts of virus-specific DNA (Fig. 9). A strongly positive signal was detected with primer pair MU3/MR, predicted to amplify DNAs whose synthesis just followed the first template switching (lane 2). In contrast, no product was obtained with primers MU3/M5NC, which flank the PBS and therefore could detect only molecules made after the second

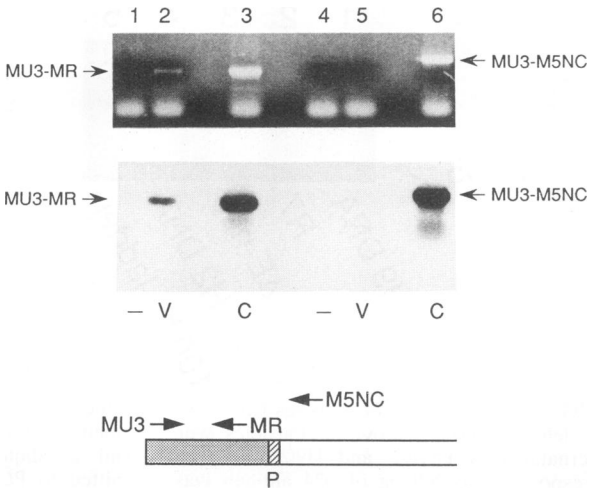

FIG. 9. Demonstration of partial reverse transcripts in MLV particles. Supernatant from the amphotropic murine retrovirus-infected cell line CEM-1B was purified by filtration and ultracentrifugation. The viral pellet was submitted to PCR amplification, with primers specific for early (MU3/MR [lanes 1 to 3]) or late (MU3/M5NC [lanes 4 to 6]) reverse transcripts. (Top) Ethidium bromide-stained agarose gel, (middle) Southern blot hybridization; (Bottom) location of primers and primer-binding site (P). Lanes: 1, uninfected cell DNA (–); 2 and 5, virions (V); 3 and 6, CEM-1B DNA (C).

jump (lane 5). This showed that in MLV, as in HIV particles, early RT products were far more abundant than late RT products.

DISCUSSION

The experiments described here demonstrate the presence of viral DNA in particles from two distinct retroviruses, HIV and MLV. This DNA is not caused by contamination by genomic DNA from infected cells but rather results from reverse transcription of viral RNA in newly assembled particles, because of the following: (i) it is resistant to DNase treatment of intact virions and becomes sensitive once these are disrupted, (ii) there is an excess of early versus late reverse transcripts, and (iii) the amount of DNA present in virions is reduced by treating the producer cells with AZT. These results are corroborated by finding virus-specific DNA associated with HIV-1 RT in preparations obtained by immunoprecipitating viral particles with anti-RT antibodies (16).

What proportion of virions contain DNA cannot be determined precisely by the technique used here. First, it is likely that a large fraction of the retrovirus-associated DNA molecules are single stranded, whereas a smaller fraction are double stranded; this complicates the comparison to an appropriate DNA standard. Second, these molecules are very heterogeneous in size; this results in an excess of templates which can hybridize to only one of the two primers used for amplification; evaluating the consequences of this imbalance on the yield obtained by PCR is difficult. Finally, RT products shorter than 139 nucleotides would fail to hybridize to the earliest primer pair R/U5 and therefore escape detection by the technique used here. Yet, virus-specific DNA was detected in HIV-1 preparations containing approximately 0.01 pg of p24 antigen (Fig. 3 and data not shown). It is estimated that each HIV-1 virion contains about 1,500 U of the core proteins, that is, approximately 6 $\times 10^{-5}$ pg of p24. On the basis of this calculation, by using the R/U5 primer pair, a PCR product can be recovered from only 1 of 6,000 HIV-1 particles. However, a number of virions might contain reverse transcripts too short to be amplified, and other particles might harbor no genomic RNA. When disrupted virions were first incubated with deoxynucleoside triphosphates in the presence of magnesium chloride to trigger an endogenous RT reaction and then analyzed by PCR with the R/U5 primers, the PCR signal was found to increase approximately 10 times (data not shown). Still, it cannot be assumed that all RNA genomes were successfully transcribed in this experiment. Interestingly, the amount of virus sufficient to detect specific DNA from HIV-1 virions by PCR is still below the minimal infectious dose for most cell types, in vitro (28a). This implies that at least in tissue culture, an infectious inoculum probably always includes some particles which have initiated reverse transcription.

These experiments are relevant for the interpretation of studies which use PCR amplification of DNA from freshly infected cells, specifically, to assess retrovirus entry and reverse transcription. The results presented here suggest that a positive signal might result from viruses bound to cells and possibly internalized but failing to pursue further their replication. Preliminary data obtained by incubating HIV-1 virions with human CD4-positive mouse cells, which can bind the virus but cannot be infected, indicate that this is the case (36). The PCR detection of virus-specific DNA in T cells incubated with HIV virions that had previously been heat inactivated and treated with DNase (37) is yet another illustration of that phenomenon.

The finding that MLV particles also contain partial RT products is important. It might otherwise have been envisioned that the presence of virus-associated DNA is unique to lentiviruses, possibly resulting from the function of a gene not present in classical retroviruses, and participated in the particular biological behavior of viruses like HIV. It is likely that most retroviruses can initiate reverse transcription at the time of assembly or shortly thereafter. Yet, the absence of intracellular buildup of viral DNA in infected cells demonstrates that few, if any, completed reverse transcription products are generated at that stage. Why? As virions are released, the viral protease cleaves the precursor for the core and enzyme proteins into individual peptides. This processing event, however, is not an absolute requirement for reverse transcription, because both the HIV precursors and the MLV Gag/Pol precursors have RT activity, at levels approaching 30 to 50% of the levels of their respective cleaved products (6, 12, 21). More simply, it is possible that viral DNA does not accumulate in producer cells because the various factors involved in reverse transcription reach a critical concentration only at the late stage of assembly, shortly before particles are released; this would leave little time for DNA synthesis.

The data presented here also imply that initiation of reverse transcription does not appear to require a structural modification of the viral ribonucleoprotein complex at the time of uncoating. Yet, the fact that the bulk of proviral DNA synthesis takes place after virus internalization remains. This might be due to the need for cellular factors, such as the nucleotides used as substrates. Whether any DNA synthesis occurs while virions are not associated with cells cannot be inferred from this work.

To what extent do particles which initiate reverse transcription before entering target cells participate in the viral infectious cycle? Other investigators have failed to demon-

strate that the biological activity of bromodeoxyuridine-substituted Rous sarcoma virus virions was photosensitive (15). In the experiments presented here, although HIV-1 particles purified from cells grown in AZT did contain lower levels of the late RT products (Fig. 6), they were not found to be significantly less infectious than viruses recovered from mock-treated cells (data not shown). However, this evidence is far from conclusive: the likelihood that viral replication is blocked by AZT (or by bromodeoxyuridine and light treatment) depends on how much DNA synthesis takes place in the presence of the drug. A majority of RT intermediates found in virions are short, only a fraction of them extending beyond the minus-strand strong stop, which in HIV-1 is located 180 bases from the primer (Fig. 5). It is important that no effect of AZT, at the concentration used, was observed on the amount of early RT products detectable in virions (data not shown). A similar finding was made by Zack et al. (37), who failed to observe a block in the synthesis of such early products, in T cells treated with 10 μM AZT plus 5 μM of another RT inhibitor, d4T (2′,3′-didehydro, 3′-dideoxythymidine). It is therefore likely that for most particles, reverse transcription stops spontaneously before AZT is incorporated and can resume normally once virions have entered target cells. Thus, until it is demonstrated that all viral DNA synthesis taking place before entry can be irreversibly blocked, without affecting the infectivity of retroviruses, one must consider that the partial reverse transcripts found in virions have an important function in the retrovirus life cycle.

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